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OBSERVATIONS ON NATURAL IMMUNITY AND SUSCEPTIBILITY TO DIPHTHERIA TOXIN.*

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THE RESISTANCE OF THE WHITE RAT.
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THE RESISTANCE OF THE WHITE RAT.

ALTHOUGH frequent mention of the high degree of immunity displayed by the rat to diphtheria toxin is to be found in the literature of antitoxic immunity, there have been but two attempts to ascertain its extent. Roux and Yersin[1] in 1888, working with black-and-white rats, observed no effect from the subcutaneous injection of ten times the guinea-pig lethal dose, but pursued the subject no further. Cobbett,[2] some ten years later, using both white and black-and-white

TABLE I.
SUBCUTANEOUS M. L. D. OF DIPHTHERIA TOXIN FOR THE WHITE RAT.

Rat No.	Weight Gms.	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses† Injected	No. Days Survived
1	113	0.0348	10	3.00	10	‡
2	137	0.42196	10	3.00	100	‡
4	150	2.31	10	3.00	500	‡
5	147	1.176	42	3.00	800	‡
18	108	1.728	45	2.73	1,000	‡
19	132	3.168	45	4.17	1,500	6
38	200	6.45	45	7.45	1,875	9
20	120	3.84	45	4.84	2,000	6
36	140	4.816	45	5.80	2,000	5
9	140	3.60	42	4.10	2,500	5½
31	206	8.838	45	9.83	2,500	6
28	130	6.71	45	7.71	3,000	5
12	177	6.195	42	7.20	3,500	4½
13	166	6.225	42	7.22	3,750	5½
14	120	4.50	42	5.50	3,750	4½
15	157	6.084	42	6.70	3,875	6½
6	144	5.76	42	6.70	4,000	3½
7	142	11.36	42	13.00	8,000	1½

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† The word gram-dose is used here to signify one guinea-pig M. L. D., calculated per gm. of the anima under discussion. In other words, one gram-dose is a dose of toxin containing 1/250 of a guinea-pig M. L. D. for each gm. of weight of the animal injected.

‡ Animal survived entirely.

rats endeavored to determine the subcutaneous dose just sufficient to kill in four or five days, and observed that, weight for weight, it was "from 1500–1800 times as great as those which suffice to kill guinea-pigs of 250 grams."

Inasmuch as the experiments planned required a knowledge of the exact minimum lethal dose for white rats, it was thought advisable as a first step to attempt a verification of Cobbett's results and to this end a large series of white rats were injected subcutaneously with varying quantities of diphtheria toxin with the results recorded in Table 1. If death on the fourth day following the injection is taken as the criterion, the data show that, weight for weight, the minimum lethal dose for the white rat averages about 3,500 times as much as for the guinea-pig of 250 gms., a figure considerably higher than that obtained by Cobbett.

This investigator used rats ranging in weight from 75 to 115 gms. and averaging not over 100 gms., while the animals used here were considerably larger, as can be seen from the recorded weights; and although in both cases the dose of toxin was corrected to the weight and calculated per gm. thereof, the discrepancy in the two results may nevertheless be due to this point. Assuming, then, that both Cobbett's results and these are correct for the animals experimented upon, the inference is that the tissues of the grown or half-grown rat are, gram for gram, about twice as resistant to this toxin as those of the quarter-grown rat, such as Cobbett used. Upon testing this hypothesis experimentally, the results from a small series of animals divided into pairs, each consisting of one rat weighing not over 100 gms. and one-half or full-grown animal, each pair being given the same dose of toxin per gm. of body weight, appear to bear out the above (Table 2). While this point is scarcely of much importance,

TABLE 2.

RELATION OF RESISTANCE TO AGE IN THE WHITE RAT.
Subcutaneous Injection of Diphtheria Toxin.

Series No.	Rat No.	Weight Gms.	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses Injected	No. Days Survived
I....................	38	200	6.45	45	7.45	1,875	9
	39	88	2.838	45	3.84	1,875	4½
II....................	28	130	6.71	45	7.71	3,000	5
	22	95	4.56	45	4.56	3,000	2½

since it is well known that very young animals are more susceptible to infectious diseases, as a rule, than older ones, the topic has been brought into this paper partly to offer an explanation of the discrepancy between the results of Cobbett and those in Table 1, and partly because of the striking manner in which this phenomenon is displayed and the interesting ratio existing between the resistance of the grown and half-grown rat and that of the quarter-grown animal, i. e., about two to one, and the length of the period of survival after the administration of a lethal dose.

In all experiments, unless otherwise indicated, the toxin was introduced beneath the skin of the abdomen by means of a Hitchens[3] reservoir syringe, which insures the injection of all the toxin measured out. To reduce the element of error arising from daily fluctuations in weight, all animals were fed and weighed at definite hours, and the dose given was always calculated per gm. of body weight. When a considerable quantity of toxin was to be injected, as was frequently the case with rats, the syringe containing the toxin was first placed in the thermostat to warm it to the body temperature, in order to lessen the shock to the animal, which even then was considerable, and in some cases so severe as to cause death, after some hours in a peculiar state of helplessness accompanied by an ague-like clonus of the skeletal muscles. While this phenomenon always accompanied the administration of large doses, it is not specific, being occasioned by large quantities of physiological salt solution as well. The toxins employed were accurately-standardized in respect to the standard-weight guinea-pig M. L. D., and were tested monthly for deterioration.

Cobbett[2] states that the tissues of the white rat are little affected by large doses of diphtheria toxin, and that he was unable to find evidence of necrosis. The autopsies performed in the course of these experiments corroborate this. As is well known, the postmortem appearances in guinea-pigs dead of diphtheritic intoxication consist of a fibrinous exudate and ecchymoses at the site of injection, enlarged and hemorrhagic adrenals, and serous exudates into the pleurae. None of these occur in the rat, and, in fact, no lesion was found that could be considered as characteristic of the administration of diphtheria toxin. While, then, no characteristic lesion is apparent, a sublethal dose even as small as 500 times the guinea-pig M. L. D. per gm. of the rat's weight causes a complete alopecia, which varies in extent from covering a circumscribed area at the injection site to almost the whole ventral surface, according to the quantity of toxin employed, the number of injections, and the interval between them. This lesion cannot be considered absolutely specific to diphtheria toxin, however, as even physiological salt solution may cause it, though to but a slight degree. Thus, to cite one instance, a rat was given 1.7 c.c. of toxin containing one thousand toxic gram-doses plus 1 c.c. of physiological saline solution. Four days later the first stages of the alopecia were observed. On the tenth day it had reached its maximum, covering the entire ventral surface from the inguinal region to the neck, and on the sixteenth day the animal had completely recovered. A second rat which had been given 10 c.c. of physiological salt solution at the same spot developed a small bare patch on the third day which reached its maximum three days later when its dimensions were about 2×0.5 cm. and which disappeared by the tenth day. A third rat given two and one-half times the quantity used for the first rat but of sterile infusion broth, containing no toxin, developed only a slight suggestion of alopecia. While the alopecia following the injection of salt solution or broth is undoubtedly due to traumatism, it seems as though the toxin must have some further action to account for the markedly greater lesion resulting from the injection of a smaller quantity of liquid. Further, the phenomenon occurs to some extent after intraperitoneal as well as subcutaneous injection of toxin but not of salt solution or broth. This alopecia has not been observed in guinea-pigs injected subcutaneously, but has been seen once or twice in rabbits.

From one to three days before death, rats frequently show a peculiar tendency to keep one or both hind-legs strongly flexed. This condition is not a true paralysis, such as is seen in guinea-pigs from the effects of late diphtheritic intoxication, for upon strong stimuli the animal is able to extend the limb. Another point of difference is that here the flexors predominate, while in the guinea-pig it is the extensors. Nevertheless, it may be analogous to that of guinea-pigs in that it is due to the presence of toxone in the toxin, because these pareses were quite common with one toxin but wholly lacking with another.

To determine the minimum lethal dose of diphtheria toxin for rats by intraperitoneal administration, another series was injected in this manner with the results compiled in Table 3, which indicate that the intraperitoneal M. L. D. is about 1,200 times as great as that for the standard-weight guinea-pig subcutaneously, corrected to the rat's weight, or, to express it more conveniently, is about 1,200 gram-doses.

TABLE 3.

INTRAPERITONEAL M. L. D. OF DIPHTHERIA TOXIN FOR THE WHITE RAT.

Rat No.	Weight Gms.	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses Injected	No. Days Survived
27	142	5·86	45	6.86	2,400	3½
24	153	5·79	45	6·79	2,200	4
25	164	4·98	45	5·98	1,800	3½
45	170	4·386	45	5·38	1,500	3½
49	242	5·9145	46	6.90	1,300	3½
55	155	3·4969	46	4·50	1,200	4
44	115	1·978	45	3·00	1,000	*

*Animal survived entirely.

The great discrepancy between this figure and that for subcutaneous injection (3,500 gram-doses) appears to be due to one of the following three causes: (*a*) In spite of the absence of connective-tissue necrosis at the injection site, the toxin is bound by the connective tissue cells. (*b*) The surplus toxin is held mechanically in the meshes of the connective tissue, the cells of which having no affinities for the toxin are immune to it, and is there either gradually transformed into some inert substance, or, more probably, held for a time and yielded so slowly to the circulation that the amount is insufficient at any one time to cause symptoms of intoxication, since, as will be shown later, the tissues of the rat do not store up small quantities of toxin giving rise to the so-called "cumulative" effect. (*c*) The other possibility is that the surplus toxin is bound by epithelial cells which, in the case of subcutaneous injection, would be those of the hair follicles, sebaceous and sudorific glands, since their anatomical distribution causes them to be the first to come in contact with the toxin.

In favor of the second view, mechanical retention by the connective tissue, is the fact that, as will be shown later, toxin remains in the connective tissue at the injection site for a comparatively long time, while the third hypothesis, epithelial binding, is supported by the fact that alopecia develops in areas, as around the axillae and neck, where it can be shown no toxin is held in the subjacent connective tissue. Indeed, the marked alopecia following subcutaneous injection of toxin is one of the strongest points in favor of the third theory. To be sure, physiological salt solution likewise produces an alopecia, but one very restricted in extent, and it is but reasonable to expect that the local traumatism resulting from the injection of 8 or 10 c.c. of liquid would suffice to interfere with the nutrition of the epithelial elements at the site and cause their atrophy. That something more than this is concerned in the case of toxin injections is shown by the far greater extent of the alopecia resulting from injection of toxin consisting of but a fraction of the control volume, and which even reaches, as stated above, to areas where the connective tissue contains no demonstrable toxin. On the other hand, however, is to be mentioned the occurrence of abdominal alopecia following intraperitoneal injections of toxin, and its appearance after the injection of a sublethal intraperitoneal dose shows it cannot be the chief agency to which is due the difference between the subcutaneous and the intraperitoneal lethal doses. In this case probably but a trifling amount of toxin brought there by the blood stream damages the hair follicles sufficiently to cause the alopecia. Why it appears only at or around the point where the needle was introduced is, however, not clear.

ARTIFICIAL EXALTATION OF THE WHITE RAT'S IMMUNITY TO DIPHTHERIA TOXIN.

Kuprianow[4] succeeded in obtaining an artificial antimicrobic immunity to diphtheria bacilli in gray rats, due to the formation of antibodies which he found in the serum, but the exaltation of the natural antitoxic immunity of the rat has been attempted previously only by Behring,[5] who failed in spite of the most careful increase from small to larger doses.

To test the possibility of producing an artificial increase in the rat's immunity, an animal was given a subcutaneous injection of 500 gram-doses of diphtheria toxin which was followed at intervals of a week or 10 days, when the animal's weight had returned to normal, by doses of 1,000, 2,000, and then 4,000 gram-doses making four injections altogether. The animal died seven and one-half days after the administration of the last dose. As the last dose was somewhat in excess of the rat M. L. D. which kills in four days as determined above, the period of survival in this case may be held to indicate either individual variation, or the acquisition of a small degree of additional resistance to the toxin. A second animal was then started with approximately weekly injections of 500, 1,000, 2,000, 2,800, 3,400, 3,800, 4,000, 4,200, 4,500, and 5,000 gram-doses in the above succession. It died one week after the administration of the 5,000 gram-doses dose from shock following the injection of 5,800 gram-doses. In this experiment the results are unequivocal and the acquisition of additional immunity is evident. During its course the animal gained about 18 per cent in weight, and finally withstood the injection of nearly 50 per cent more than the four-day fatal dose with no appreciable signs of disturbance, the weight remaining practically stationary during the succeeding week. As the animal's death was due, not to intoxication from the last dose of 5,800 gram-doses, but merely to the shock from the injection of so large a quantity of liquid (about 11 per cent of the body weight), it is impossible to say to what degree this artificial immunity might be raised. Apparently the only check on an indefinite increase by means of small gradations as above is the volume of liquid that can be introduced subcutaneously without producing fatal shock. Experiments are at present under way to test this hypothesis as well as to see if the serum of an animal, immunized in this manner, possesses any antitoxic powers.

It is interesting to note that the artificial immunity thus acquired is transitory in nature and persists less than one month if injections are withheld.

HAS DIPHTHERIA TOXIN A CUMULATIVE ACTION TOWARD THE WHITE RAT?

Certain drugs like mercury, lead, the iodides, and arsenic, as is well known, if administered in small doses over a long period, have a much more strongly poisonous effect than if the sum of all these doses be given at once. This is held to be due to an accumulation in the tissues of a fraction of each dose administered which eventually has increased to the lethal quantity, while from a single large dose (e. g., lead) not enough is absorbed to produce symptoms. The exhibition of such a cumulative action by diphtheria toxin upon the rat would help to explain the latter's high resistance toward this toxin, as well as show whether the toxin can be stored up in the tissues of the rat if given in sufficiently small doses. To determine this point the following experiment was performed:

A rat was given eleven injections of 1,000 gram-doses, five injections of 2,000 gram-doses, and then one injection of 3,000 gram-doses; whereupon the experiment was interrupted by the pregnancy of the animal. The rat had nevertheless received, in the course of about two and one-half months $\frac{24,000}{3,500}$, or approximately seven times the lethal dose, and instead of showing any ill effects from the treatment, gained weight, carried through a successful pregnancy, and even acquired some degree of artificial immunity, as was shown by the progressively smaller disturbance following the injections, although the latter were twice increased. Further experimental evidence of the absence of cumulative action on the rat by this toxin is afforded by the immunization experiments described above.

The absence in the rat of the phenomenon of hypersusceptibility displayed by the guinea-pig after the injection of a number of small doses is demonstrated by another experiment. A rat was given 38 daily injections of 100 gram-doses, or $\frac{1}{35}$ of the rat M. L. D. each, on five days in each week, so that at the end of the eighth week the animal had received $\frac{38}{35}$ M. L. D., or somewhat more than the fatal dose, but distributed over two months. The fact that the rat lived and even gained weight during the experiment shows that hypersusceptibility is not concerned in the reactions of the rat to diphtheria toxin.

THE FATE OF DIPHTHERIA TOXIN IN THE BODY OF THE WHITE RAT.

The advances that have been made along the line of antitoxic immunity have been accomplished almost wholly through study of the artificial type thereof. That a clearer conception of natural immunity would go far toward the solution of the problems of antitoxic immunity in general will scarcely be disputed, but up to the present time very little work has been done in this field. While a few researches have been published dealing with the fate of tetanus toxin in certain of the lower forms, hardly anything has been done in regard to the mammals, notwithstanding the prime importance of a knowledge of the mammalian reactions. In the hope of throwing a little light upon certain phases of mammalian natural antitoxic immunity, the following experiments were undertaken.

Roux and Borrel[6] have shown that, in spite of the high resistance of the rat to diphtheria toxin, the animal is extremely susceptible when the toxin is injected intracerebrally, 0.1 c.c. producing death in two or three days; hence the natural immunity displayed toward other modes of injection must rest upon the failure of the toxin to reach the brain. Behring[5] supposed that this was due to a "special property of the cell-free blood," i. e., antitoxic principles in the serum; while Cobbett[2] states that the serum of white rats in quantities of 1 c.c. does not protect guinea-pigs from a little over the minimum lethal dose of toxin. The two views are thus diametrically opposed. In order to determine this point, a guinea-pig of test weight was injected with a mixture of the M. L. D. of toxin and 0.6 c.c. of normal rat serum. The latter afforded not the least protection. As it is possible that antitoxin does appear in the rat's blood but is generated only by the stimulus afforded by the presence of toxin, a second rat was injected with 800 toxin gram-doses subcutaneously, and the above experiment repeated after the lapse of a week, with the same result. The objection may be raised here that inasmuch as 800 gram-doses are less than the difference between the subcutaneous and the intraperitoneal rat M. L. D.'s and therefore within the limits of the amount of toxin taken up by the connective tissue or epithelial structures at the injection site, too small a proportion of the body cells are exposed to the stimulus afforded by proximity of toxin, and thus only an inappreciable amount of antitoxin is formed and finds its way into the blood stream. While this objection has some weight, still the toxin if not actually bound chemically by cells at the site must be eliminated some time, and if it is yielded to the circulation slowly and in small quantities, the long continued exposure to it of all the body cells ought to afford excellent opportunities for antitoxin formation. An experiment was performed, however, to settle the question by examining the serum of a rat subjected to intraperitoneal administration of a sublethal dose (1,000 gram-doses). It was found that 1 c.c. of such serum two weeks after injection afforded no protection, so that we are justified in concluding that the rat's immunity is not due to circulating antitoxins. The presence of antitoxin in the blood of rats which have been actively immunized against the toxin is, of course, another question and has no bearing, strictly speaking, upon natural immunity. Behring bases his belief that the immunity of rats is due to antitoxin in the blood upon an experiment in which he injected large doses of diphtheria toxin into rats, intraperitoneally, and then after three hours bled them and injected 4 c.c. of their serum into guinea-pigs, also intraperitoneally. The latter showed no signs of intoxication, although the same quantity of serum of animals susceptible to this toxin gives distinct signs of intoxication in guinea-pigs, the same procedure being observed; and the blood of immunized guinea-pigs gives the same results as that of normal rats. The flaw in this reasoning is obvious. Absence of toxin in the blood three hours after injection is, at least, just as probably due to its failure to get into the blood stream at all, as to the presence of specific antibodies there to neutralize it.

The next step was to attempt to follow the toxin throughout its sojourn in the rat's body from the time of injection until its elimination in the urine, which Cobbett[7] has shown to take place. The only other work of this nature upon a naturally immune animal is the experiment of Brunner[8] on dogs, in which he tested the blood and a compound emulsion of the liver, spleen, kidneys, and muscle of an animal killed after a toxin administration, by injection into other dogs. The blood, given intravenously, yielded negative results, while the tissue emulsion, given intraperitoneally, killed in 18 hours.

Although the literature upon the fate of diphtheria toxin in immune animals is so scanty, there are a few researches in regard to the fate of tetanus toxin in some of the lower animals that are naturally immune to it, and for purposes of comparison it may be worth while to cite them briefly.

Asakawa[9] and Vaillard[10] found that tetanus toxin persists for five or six days in the blood of the chicken. The latter has shown further that the sexual glands alone of all the viscera absorb some of the toxin; no antitoxins occur in the blood. Metchnikoff[11] has proven that this persistence in the blood is due to the absorption of a portion of the toxin by the leucocytes. In regard to the poikilothermous species it is reported by the same investigator[12] that in the alligator the toxin is rapidly eliminated from the blood even when kept at relatively low temperatures (20° C.) though in the marsh-turtle the toxin passes quickly into the blood and remains localized there for more than four months. Two months after injection of 500 mouse M. L. D.'s the blood of a green lizard kept at 20° was still extremely toxic while in the scorpion the toxin remains in the blood for but a few days passing thence to the liver only and remaining there unaltered for a month or more. Green frogs, again maintain the toxin in their blood for at least two months.

To trace diphtheria toxin through the rat's body, a series of guinea-pigs were given subcutaneous injections of suspensions of the various tissues of rats killed at different intervals after the administration of a sublethal dose of toxin (3,000 gram-doses) subcutaneously.

The suspensions were prepared by grinding up the tissue with physiological salt solution in a sterile mortar, straining through cheese-cloth to get rid of the reticulum and finally diluting with salt solution up to 4 c.c. The amount of each tissue used was not weighed each time but as close an approximation as possible to the same volume of a tissue was used in each experiment. Of the blood 1.25 c.c. were injected. This quantity would contain about 15 guinea-pig M. L. D. if the toxin were uniformly distributed throughout the rat's body. Of the other tissues about 1.7 gm. of brain (entire viscus) were injected, which would have a toxin equivalent of 21 M. L. D.; of connective tissue 0.25 gm. (3 M. L. D.); of kidney (one viscus) 0.7 gm. (8.5 M. L. D.); of muscle (right and left pectoralis major) 2 gm. (24 M. L. D.); of liver 1 gm. (12 M. L. D.); of spleen 1 gm. (12 M. L. D.); and of adrenals (both) 0.045 gm. (0.75 M. L. D.). Inasmuch as the injection of normal rat tissues gives rise to abscesses and eventually ulceration in guinea-pigs, whereby fatal infections were often incurred, acute death within the arbitrary limit of one week was taken as evidence of the presence of toxin while survival beyond that period was held to indicate its absence in the tissue examined. It may be mentioned here that this division was easily made as there were no deaths in the entire experiment between the sixth and the fifteenth day after injection. In all instances the typical postmortem picture of diphtheritic intoxication was displayed by the animals dying during the first week. In rather less than half the acute cases the gastric or duodenal hemorrhages of Rosenau and Anderson[13] were observed. In the 18-hour series in addition to the routine tissues tested and recorded, the lungs, thymus, myocardium, lymph glands, and stomach wall were also tried, with negative results.

Upon referring to Table 4 the history of the toxin injected subcutaneously is readily traced. Eighteen hours after injection part of

TABLE 4.

LOCATION OF DIPHTHERIA TOXIN IN BODY OF WHITE RAT AT VARIOUS INTERVALS AFTER INJECTION.

TISSUE	NO. HOURS AFTER INJECTION							Normal
	18	24	48	72	96	120	168	
Adrenals	−	−	−	−	−	−	−	−
Blood	+	+	+	−	−	−	−	−
Brain	−	−	−	−	−	−	−	−
Connective tissue (site)	+	+	+	+	+	+	+	−
Connective tissue (axillae and neck)	−	+	+	−	−	−	−	−
Kidney	−	−	+	+	−	−	−	−
Liver	+	+	+	+	+	−	−	−
Muscle	−	+	+	−	−	−	−	−
Spleen	−	−	−	−	−	−	−	−

the toxin has found its way from the connective tissue interstices at the injection site to the blood stream, and has been taken up from the latter by the liver alone of the viscera. Six hours later it appears in appreciable quantity in the general connective tissue of the body and in the skeletal muscles, while 24 hours after this it is to be found most generally diffused over the body, the brain and spleen and adrenals alone being free from it. In another 24 hours it has disappeared from the blood, muscle, and general connective tissues, and on the following day the kidney fails to show the toxin. On the fifth day after the injection the toxin abruptly disappears everywhere except from the original injection site, where it can be detected for seven days altogether.

Thus when a sublethal dose is injected under the skin of the rat, a part remains unchanged in the connective tissue at the injection site for a comparatively long period. The remainder soon gets into the blood stream, undoubtedly by way of the lymphatics. From the blood it is gradually taken up by the various tissues until 48 hours after the injection when the toxin is diffused generally over the body. After this period a receding from these organs and tissues occurs, until on the fifth day it can only be detected at the original site. It is interesting to note that after the period of maximum diffusion (48 hours), the toxin disappears from the blood though persisting in other tissues. It suggests that the accumulation of toxin in the connective tissue at the site is not dissipated by being surrendered gradually to the circulation and thence excreted by the kidney, as is the ordinary physiological procedure, or by the liver, as is the usual fate of toxic substances, but that it is more probable we have to do here with a rather considerable discharge into the circulation of the toxin in excess of what can be accommodated by the local connective tissue. The surrendered portion circulates in the blood, and, being brought into intimate relation with the various tissues, is taken up by them. This is supported by the results of a similar series of experiments in which the toxin, 1,100 gram-doses, was introduced intraperitoneally so as to eliminate the influence of the connective tissue. After 24 hours the toxin could be found in the liver alone of all the tissues, and it had disappeared thence after 48 hours. The rapidity of the disappearance of the toxin in this case is undoubtedly due to its being surrendered to the blood far more rapidly by the peritoneum than by the subcutaneous tissue. This would explain the disappearance of the toxin from the blood after the period of maximum diffusion in the body, when it might be supposed that all the toxin in the blood is removed from it by the other tissues, but it complicates the question of elimination. As mentioned above it has been shown that diphtheria toxin is excreted in the urine of the rat, at least a few hours subsequent to injection. It is easily seen how a considerable portion of the toxin can thus be eliminated during the period in which the toxin is to be found in the blood. But what is the mechanism in regard to the elimination of that portion remaining in certain tissues after the blood ceases to contain toxin? Is it yielded to the blood and thence to the kidneys so gradually and in such small quantities that even so delicate an indicator as the guinea-pig biological test is insensitive to it, though huge doses of toxin are given the rat; or do the tissues in which it is held break it down or build it up into an inert substance? The absence of antitoxin in the rat's blood by no means precludes the possibility of an intracellular neutralization, although at present we have no definite evidence of such an occurrence. If the former hypothesis of gradual elimination be true, it might be possible to get experimental evidence by injecting a guinea-pig with a large quantity of rat's blood in the hope that if only a small quantity of toxin be in the blood, the amount of blood used would bring this toxin modicum up to the guinea-pig M. L. D. Of course while a positive result would prove the case, a negative result could throw no light upon the subject either way, for the amount of toxin, if present, might be sublethal even in the larger quantity of blood. The latter was the fate of an experiment of this kind. A guinea-pig was injected with 6 c.c. (about five times the quantity usually used in these experiments and which would contain about 75 guinea-pig M. L. D. if the toxin were uniformly distributed) of the blood of a rat killed 96 hours after the toxin was injected. This period was chosen because then the process should be most marked since at this time the elimination of toxin from the various tissues is at its height. The animal survived the test period, living three weeks, and thus giving no information. Larger quantities of blood than 6 c.c. were not employed because the injurious effect (ulceration) of normal rat's blood itself is so pronounced as to invalidate conclusions.

Regarding the other tissues it might appear that the toxicity of suspensions of a such highly vascular organ as the liver for example, is due merely to the amount of blood it happened to contain at the time. While this is probably the case to a considerable extent, and particularly true of the liver in the earliest stages, the fact that the toxin is evident there some time after disappearing from the blood shows that the cells themselves play an important rôle, and that, at least in the later stages, it is the tissue and not its blood content that gives the reaction. The fact that the liver contains toxin at all periods up to the fifth day, and is the only tissue (site excepted) so doing, might be regarded as a hint that it has something to do with the transformation or elimination of toxin. But if so, how does the toxin taken up by other tissues reach the liver after its disappearance from the blood ?

As for the kidney, the absence of toxin there before the second day apparently does not agree with Cobbett's statement that the toxin is eliminated by the urine, but the small amount of the latter present in the kidney is insufficient to cause symptoms even when it contains toxin, while the results for the second and third day show that even by that time the kidney tissue itself has absorbed some toxin from its blood-supply. The results with the spleen, showing the absence of demonstrable toxin at all stages, are difficult to explain in view of the great vascularity and enormous blood content in proportion to its own weight, as it would seem that this alone would cause intoxication. Of course in testing the blood a larger quantity was employed than was contained in the bit of spleen, and it is possible that this latter amount was just too small to contain a fatal dose.

The direct or "in vitro" power of the spleen, as well as of the myocardium, liver and brain, to neutralize toxin was tested* after the method of Wassermann and Takaki.[14] It was found that these tissues were unable to bind even two M. L. D. in such a manner as to protect a guinea-pig injected with the triturated emulsion, although even with a uniform distribution of the toxin throughout the rat's body each gram of body tissue should be capable of neutralizing about 12 M. L. D., as indicated above. The effect of these tissue-triturate emulsions and toxin was the same whether the tissue was ground with the toxin or the expressed fluid mixed with the toxin after grinding, and whether the mixture was kept at 20° C. or 37° C. for 30 minutes previous to injection. It is evident therefore that the living cells and not merely the tissue substance are necessary for the absorption of the toxin. These living cells do not include the leucocytes which, it will be remembered, were found by Metchnikoff to take up tetanus toxin in the fowl. The point is proven by the following experiment:

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* In the experiments testing the effect of mixtures of tissue emulsions and toxin to determine the existence of an "in vitro" neutralizing power by these viscera I was assisted by Mr. C. D. Enfield, a student in this laboratory.

A normal rat was bled into a sterile 1 per cent solution of sodium citrate in 0.85 per cent sodium chloride solution to prevent coagulation. The mixture was centrifugalized and washed twice in sterile physiological salt solution to remove traces of citrate, the supernatant liquid removed and 1.5 c.c. of the upper layer of corpuscles taken as leucocytes. To these were added 2 M. L. D. of toxin and enough normal salt solution to make the whole up to 4 c.c. This was incubated at 37° for 30 minutes and then injected into a guinea-pig. No protection was afforded. Since each gram of leucocytes would receive about 12 M. L. D. if the 3,000 gram-doses of toxin used in the above experiments had been uniformly distributed throughout the rat's body, and since they would be able to accommodate many times that quantity if the leucocytes are the real protective agency as in the case of the fowl, failure to protect against 2 M. L. D. indicates that the leucocytes are not to be considered in the resistance mechanism.

Besides the spleen, the brain is the only tissue which at no stage gives evidence of containing toxin. The subject is simpler here than in the case of the spleen, for the rat's extreme susceptibility to intracerebral injection of diphtheria toxin, shows that if any were to reach the brain the animal would quickly succumb. Indeed the entire problem of explaining the immunity of the white rat to the subcutaneous injection of toxin is expressed in the question why the toxin does not reach the susceptible central nervous system. The old theory of Ehrlich's school, that natural immunity is due to a scarcity of suitable receptors to bind the toxin, is quite inadequate, for (*a*) if there are no receptors capable of combining with the toxin, it would not be possible to kill the animal, no matter what the dose used, and (*b*) if there were but few suitable receptors it would require a smaller dose to saturate these and produce death than in the case of a susceptible animal which possesses a larger number of receptors. Similarly it cannot be due merely to the presence of an unusually large number of receptors in the connective tissue at the injection site which absorb all the toxin and leave only a small fraction, or none at all, to get into the blood stream, for these experiments show that toxin does get into the blood in considerable quantity, and, indeed, the results of experiments with the viscera of rats injected intraperitoneally bear out the above completely. Lastly, the immunity of the rat cannot be due to the absorption of the toxin by the leucocytes, as Metchnikoff has shown takes place in the fowl, because (*a*) the toxin persists in several tissues after having disappeared from the blood, (*b*) the pre-eminently leucocytic tissues such as the lymph glands and the spleen show no evidence of the presence of toxin, and (*c*) "in vitro" experiments with living leucocytes show them to lack the power of taking up toxin. To be sure, the lymph glands were examined only at the 18-hour stage, but the spleen was tested at each period, and further, if the leucocytes did have the same function as in the fowl, it should have manifested itself in the lymph glands at the time they were examined. The subject is thus exceedingly complex, and inasmuch as the data obtained contradict our present hypotheses, it is, perhaps, permissible to attempt to formulate a theory to explain natural immunity in the rat, basing it upon the information secured in the foregoing experiments. The hypothesis I beg to submit is that there are three types of cells in the rat in relation to diphtheria toxin, (1) those possessing no receptors capable of uniting with diphtheria toxin, and hence inert in respect to it; (2) those possessing receptors capable of uniting with it, but whose receptors (α receptors) have but a low degree of affinity for the toxin; when the latter is bound, however, the toxin enters into such a combination with the protoplasm of the cell that its vitality is destroyed. The best examples of this type are the brain cells. (3) The third type of cell also possesses receptors able to bind the toxin, but these (β receptors) differ from the α receptors of the nervous elements in that they have a high degree of affinity for the toxin molecule. Another point of difference between the cells of the second and third types is that when bound by the receptors of the latter, the toxin molecule does not enter into a combination with the cell protoplasm, which is incompatible with continued life of the latter, but is treated by the cell as a food, and after remaining in the cell a sufficient length of time is broken down to simpler bodies through the agency of intracellular enzymes, perhaps, whose function it is so to treat the various food elements taken up by the cell. Eventually part of the original toxin molecule is built up into the protoplasm of the cell, while the remainder, of course no longer to be considered as a toxin, is eliminated by the cell in the usual manner. Such a body could not be detected in the blood. Examples of cells of this last type are those of the liver, and kidneys.

Turning now to Table 4, how is it to be interpreted according to the foregoing hypothesis? When the toxin is introduced beneath the skin in sublethal doses it penetrates the interstices of the connective tissue, at the injection site, to a certain extent. The cells composing this connective tissue, belonging as they do to the first type, do not unite with the toxin in any way and remain passive in respect to it. This is shown by the following points: (*a*) They cannot belong to the second type of cells because there is no detectable necrosis to be observed at the injection site. (*b*) They cannot belong to the third type of cells because the toxin disappears from the connective tissue elsewhere in the body at the same time that it disappears from the blood, whereas it should remain there at least 24 hours after leaving the blood stream, before destruction by the intracellular enzymes. Therefore the presence of toxin in the general connective tissue at the 24- and 48-hour periods is due to mere physical diffusion, which would explain its elimination as soon as the concentration of the toxin in the blood begins to decline. (*c*) It is conceivable that these connective-tissue cells do not enter into combination with the toxin in any way, but secrete an extracellular enzyme capable of breaking up the toxin. That this is not the case, however, is shown by the long persistence of the toxin in the meshes of the connective tissue at the injection site.

A second, and much smaller, portion of the administered toxin unites with the second-type cells of the hair follicles (and perhaps sebaceous and sudorific glands) in that location, and, killing them, produces the alopecia described earlier. This alopecia is slow in appearing because of the low affinity of the *a* receptors for the toxin, and indeed the only reason it occurs at all with these sublethal doses is because of the large amount of toxin continually held there by the connective tissue meshes. The remainder of the toxin, that is, the excess beyond the holding capacity of the connective tissue, rapidly passes into the blood stream which carries it to all parts of the body. The highly susceptible brain is thus exposed as early as the other tissues, and the reason the animal is not killed is because the brain, in spite of its susceptibility, has but feeble combining powers with the toxin, its cells being of the second type, while other tissues belonging to the third type, such as the liver, etc., which are immune to the intracellular action of the toxin, have a very strong affinity for the latter's binding group and so withdraw all the toxin circulating in the blood, a process which, coupled with excretion by the kidney and liver, and possibly sweat (Salter[15]), leaves none to unite with the weak *a* receptors of the brain. The toxin taken up by tissues containing type-three cells remains intracellular for a period varying with the amount taken in and the activity of the enzymes, until the latter have so changed its chemical composition that it no longer reacts to the biological test. The end products of its katabolism, no longer toxic, are excreted by the cells into the circulation and eliminated by the kidneys, as is the case with other food elements.

To summarize, the immunity of the white rat to large doses of toxin rests upon prevention of the latter from uniting with the highly susceptible nervous tissues because of the greater affinity of other tissues composed of cells insusceptible to the intracellular action of the poison due to a difference in the chemical composition of their protoplasm. A lethal dose of toxin, then, is one which exceeds the capacity of the body's type-three cells to take up the toxin leaving the surplus to circulate in the blood until the slowly acting *a* receptors of the brain become saturated and death ensues—a case quite similar to what happens in the causation of alopecia at the injection site, or upon intracerebral injection of the toxin when the brain tissue is actually bathed in the poison.

If later experiments show that artificial exaltation of immunity is not due to the presence of antitoxins in the blood, it could readily be accounted for by this hypothesis on the basis of a greater capacity of the third type of cells to take up the toxin from the blood, due to a more rapid katabolism of the toxin in these cells because of an increased production of the intracellular enzymes concerned. It is easily recognized that such an increase in the rapidity of enzyme production is borne out by analogy with all that is known of antibody formation.

Finally it may be mentioned that what we know of the part the liver plays in the elimination of poisons in general agrees with the evidence given in these experiments of its preponderating role in the protection of the rat against this toxin.*

THE RESISTANCE OF THE RABBIT TO DIPHTHERIA TOXIN.

The only data hitherto published upon this point are contributed by Roux and Yersin,[16] who indicate that gram for gram the rabbit is twice as susceptible to the (subcutaneous ?) injection of diphtheria toxin as is the guinea-pig. Finding it necessary to confirm this, a series of rabbits were injected under the skin of the abdomen with varying quantities of toxin, death on the fourth day being taken to indicate the minimum lethal dose. The technic employed was the same as that described above in regard to rats. At autopsy it was observed that rabbits dead of diphtheritic intoxication from subcutaneous injection always show a fibrinous or sero-fibrinous exudate, usually quite abundant in amount, at the site, but no other uniform lesion. The stomach lesion observed by Rosenau and Anderson[13] in guinea-pigs could not be detected in the rabbit. There is frequently, however, a hemorrhagic condition of the adrenals, testes or ovaries and Fallopian tubes.

Examination of Table 5 shows that weight for weight the rabbit and guinea-pig are about equally susceptible to this toxin, and do not exhibit the difference stated by Roux and Yersin. The discrepancy perhaps is due to the fact that the latter observers used 400-gram guinea-pigs as standards instead of the present-day test weight. We know now that these larger animals do not exhibit the same uniform-

TABLE 5.

RABBITS. SUBCUTANEOUS M. L. D. OF DIPHTHERIA TOXIN.

Rabbit No.	Weight	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses	No. Days Survived
4	1264	.00632	42	2.4	½	+
5	1477	.01477	42	2.1	1	5½
19	1577	.02712	45	1.5	1	3½
7	1250	.025	42	3.0	2	2½
8	1205	.03615	42	2.3	3	1½

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* It was intended to carry out a similar study of the reactions to diphtheria toxin displayed by a naturally immune poikilotherm—the brown-frog. Experimental difficulties, however, compelled abandonment of the attempt. One or two of the observations nevertheless may be of interest. The brown frog, kept at low temperatures, is not affected by the intraperitoneal administration of 8,000 gram-doses of diphtheria toxin. After 24 hours injected toxin is to be found only in the blood and liver. It will be recalled that Metchnikoff observed that toxin persisted in the blood of green frogs for some two months.

ity in their reaction to toxin as those of 250 grams. Further, their rabbits were considerably larger (3 kg.) than those obtainable here, and, finally, it is quite impossible to determine the minimum lethal dose for rabbits as accurately as for guinea-pigs, partly because of the greater daily fluctuations in weight, due to variations in the quantity of food eaten at the last meal, and in the rate and time of defecation, and partly because of the greater individual variation in resistance in this species. In connection with the close equality in susceptibility to diphtheria toxin shown by the rabbit and guinea-pig, it is interesting to note the extent of the disparity existing between them in regard to tetanus toxin, to which according to Knorr[17] the rabbit is about one thousand times more resistant, gram for gram.

The intraperitoneal (Table 6) and intravenous (Table 7) minimum lethal doses of diphtheria toxin for rabbits were next determined in a similar manner. Comparison of Tables 5, 6, and 7 shows that the rabbit is about three times as susceptible to this toxin when it is administered intravenously or intraperitoneally as subcutaneously. In other words the tissues at the injection site are capable of holding in some way a quantity of toxin twice as great as the true, or intravenous, M. L. D. The explanation of this phenomenon is by no means the same problem as that encountered in the case of subcuta-

TABLE 6.

RABBITS. INTRAPERITONEAL M. L. D. OF DIPHTHERIA TOXIN.

Rabbit No.	Weight	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses	No. Days Survived
61	2155	.0081	46	1.5	1/5	..
2	1735	.01185	45	1.2	2/5	2½
11	1485	.01114	42	2.1	3/4	2½
10	1625	.0079	42	3.3	1/3	6½
56	2285	.0215	46	1.5	½	1½

TABLE 7.

RABBITS. INTRAVENOUS M. L. D. OF DIPHTHERIA TOXIN.

Rabbit No.	Weight	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses	No. Days Survived
1	1264	0.00632	42	2.4	½	1½
3	1332	0.02664	42	4.5	2	½
6	965	0.004825	42	1.95	¼	6½
12	1480	0.0049	42	2.5	1/3	4½

neous injection in the rat, for while the latter belongs to the group of naturally immune mammals, the rabbit is highly susceptible by nature, and therefore each must be studied separately.

If it is the action of the local connective tissue that causes the difference between the subcutaneous and intravenous M. L. D.s, the question is raised why the great amount of connective tissue throughout the body does not protect it against the intravenous M. L. D., since the small amount of connective tissue at the injection site is able to take up in some way twice that quantity of toxin. Certainly toxin introduced into the blood is brought into intimate relation with all the connective tissue of the body, so that the latter might be expected to withdraw all the toxin from the blood and save the animal. That this does not occur indicates that the connective tissue cells are either unable to unite with the toxin at all or else do so very slowly. In either case they would be unable to afford the protection mentioned against subcutaneous injection. To be sure, the necrosis of the connective tissue at the injection site shows that these cells can unite with the toxin, but the fact that there are no focal necroses in the connective tissue after intravenous injection indicates that the selective power of the connective tissue is not great. Physical absorption seems scarcely probable because in that case the rabbit would be able to accommodate more toxin subcutaneously than the white rat, since it has a much larger amount of connective tissue, but, as a matter of fact, the rabbit is vastly more susceptible to toxin given subcutaneously. A more plausible explanation, perhaps, is that the presence of very large quantities of toxin, comparatively, is able to bring about reactions which smaller quantities cannot. Thus the presence of an entire intravenous M. L. D. or double that quantity in the subcutaneous tissue enables the toxin not only to unite with some of the connective tissue cells causing a local necrosis but so to damage the endothelium of the local blood vessels that edema occurs, the coagulation of the fibrinogen therein resulting in the appearance of the fibrinous exudate mentioned above. This exudate may be the real element of defense, and may function either physically, by holding the toxin in its meshes, mechanically, by walling off the toxin-infiltrated area and preventing diffusion of the poison into the body, or chemically, by entering into combination with a certain amount of the toxin and binding it, thereby shielding the animal; in any case, the result is a capacity to take care of twice as much toxin as the intravenous M. L. D. When more than this is given, either because it diffuses out before the mechanism is in working order or because it is too much for the local powers of accommodation, the excess gets into the blood stream where it circulates in decreasing quantities for about eight hours (de Croly[18]), or perhaps some what longer (Bomstein[19]). While it is thus brought into intimate relation with the connective tissues of the body in general, the circulating toxin is not taken up by them because of the lack of the above two factors to which are due the holding of toxin at the injection-site. In the first place, the requisite degree of toxin concentration is not afforded because of the dilution from mixture with the entire blood supply, and because only a fraction of the toxin administered reaches the blood at all. In the second place, the period of exposure to the toxin is much shorter. Thus de Croly observed that while toxin injected intravenously was present in the blood in practically the entire original amount fifteen minutes later, after two hours only from one-fourth to one-half remained, and it had practically disappeared at the end of eight hours. Bomstein obtained about the same results. After one hour he found in the blood one-half, after three hours one-quarter, and after 12 hours only one-eighth, of the amount injected. This sharp decrease in the toxin content of the blood must be due to the fixation of the toxin by some cells apparently essential to life and possessing a strong affinity for the toxin molecule. According to Bomstein no appreciable quantity of toxin is eliminated by either the urinary or the alimentary systems.

Regarding the possible modes of action of the fibrinous exudate in causing the discrepancy between the subcutaneous and intravenous M. L. D.s, according to the physical-absorption view we would assume that the toxin is taken into the meshes of the fibrin and held there so as to delay but not prevent its reaching the circulation. Practically the full amount of toxin given subcutaneously, therefore, will get into the blood stream, but as it diffuses slowly and since we arbitrarily take death on the fourth day as the criterion, the subcutaneous dose must be so large that the fraction reaching the blood during the first few days equals the intravenous M. L. D. The hypothesis of mechanical action would consist merely of a variation upon the foregoing; the fibrin of the exudate forms a wall or barrier impervious to the fluid which prevents diffusion of the toxin, after the manner of peritoneal adhesions limiting a localized infection like appendicitis, instead of acting as a sponge as would be the case if the first view were true. A toxic dose would be one large enough to permit the diffusion of an intravenous M. L. D. before the fibrinous exudate had completed walling off the injected area. It is to be noted in this case that the remainder of the toxin would never reach the circulation, as would eventually occur in the case of physical absorption. The hypothesis of chemical neutralization or binding of the toxin by the fibrin needs no explanation except that here also a lethal dose would be one large enough to permit the diffusion of an intravenous M. L. D. before enough fibrin has been formed to accomplish its purpose of neutralization.

Although, as stated above, it appears improbable that this protection is due to mere physical absorption of the toxin by the local connective tissue, after the manner of the protective action of charcoal, for example, it was thought advisable to obtain some experimental evidence upon the point. It seems reasonable that if the local connective tissue is able to absorb twice the intravenous M. L. D. or two-thirds of the subcutaneous dose, the injection at different points of two or three doses whose individual value is slightly less than this figure but whose sum exceeds the subcutaneous M. L. D. ought to have no particularly deleterious effect upon the rabbit, since at each injection-point the local connective tissue is able to hold more than the injected amount. To test this a rabbit was given three subcutaneous injections simultaneously at different points on the ventral surface, each injection equal to one-half the subcutaneous M. L. D. and therefore individually less than the difference between the subcutaneous and intravenous M. L. D. but collectively amounting to 50 per cent more than the subcutaneous M. L. D. The animal succumbed acutely in three days, showing that this hypothesis is inadequate. On the other hand, if the mechanism is either physical absorption or mechanical walling-off by fibrin, the administration of a dose large enough to induce fibrin formation but not so great as seriously to hurt the animal, and followed after four to seven days, when the fibrinous exudate is completely formed, by a second and lethal dose, should have no effect upon the animal, since its protective mechanism would be in working order. A number of experiments performed in another connection (Table 8) show that neither of these views are tenable. The conclusion then, seems unavoidable that (*a*) the protection afforded by the connective tissue is due to the formation of fibrin, and (*b*) the action of the fibrin is chemical rather than physical or mechanical.

By the hypothesis of chemical action the results of the last-quoted experiments may be easily explained, since the fibrin formed by the stimulus afforded by each injection is largely neutralized by the toxin in the process of uniting with it, and therefore another larger dose would have almost the same effect upon the animal as if no fibrin had been previously formed.

Normal rabbit serum does not protect guinea-pigs against a lethal dose of toxin, so it is evident that the presence of free antitoxin in the blood is not a factor in the mechanism of the rabbit's resistance to diphtheria toxin.

IMMUNIZATION AND HYPERSUSCEPTIBILITY OF RABBITS TO DIPHTHERIA TOXIN.

The literature contains no mention of any previous attempts to obtain an artificial immunity in rabbits to diphtheria toxin, although the subject of the possibility of artificial immunization of naturally susceptible animals to bacterial poisons is of great importance because of its bearing upon vaccination and serum therapy. As for other mammals naturally susceptible to diphtheria toxin, it is familiar that the horse, goat, and cow can be immunized readily while the guinea-pig is somewhat refractory to the process and can be immunized to but a limited extent.

In a series of experiments designed to test the possibility of rabbit immunization (Table 8), in spite of varying the size of the initial dose, the gradual increase in the amount administered at each injection and the time interval between injections, it has thus far been found impossible to diminish the rabbit's susceptibility to the M. L. D. The variations in the initial dosage ranged from $\frac{1}{8}$ to 1/10,000 of the M. L. D. (either subcutaneous, intravenous, or intraperitoneal, as the case might be), while the interval between injections varied from 1 to 14 days.

TABLE 8.

Rabbit No.	Mode of Injection	Injections in M. L. D.	No. Days Interval	Days Survived after Last Injection	Sum of All Injections in M. L. D.
27	S	$\frac{1}{1000}$, $\frac{1}{500}$, $\frac{1}{250}$, $\frac{1}{125}$, $\frac{1}{60}$, $\frac{1}{30}$, $\frac{1}{15}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1.	1	$3\frac{1}{2}$	2
28	S	$\frac{1}{100}$, $\frac{1}{50}$, $\frac{1}{25}$, $\frac{1}{12}$, $\frac{1}{6}$, $\frac{1}{3}$, $\frac{1}{2}$, 1, $1\frac{1}{4}$	1	$1\frac{1}{2}$	$3\frac{2}{5}$
33	S	$\frac{1}{100}$, $\frac{1}{50}$, $\frac{1}{25}$, $\frac{1}{12}$, $\frac{1}{6}$, $\frac{1}{3}$, $\frac{1}{2}$, 1	1–2	$2\frac{1}{2}$	$2\frac{3}{20}$
32	S	$\frac{1}{10000}$, $\frac{1}{5000}$, $\frac{1}{2500}$, $\frac{1}{1250}$, $\frac{1}{625}$, $\frac{1}{300}$, $\frac{1}{150}$ $\frac{1}{75}$, $\frac{1}{40}$, $\frac{1}{20}$, $\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{3}$, $\frac{2}{3}$	1–7	$4\frac{1}{2}$	$1\frac{2}{5}$
29	S	$\frac{1}{10}$, $\frac{1}{8}$, $\frac{1}{6}$	3–4	$14\frac{1}{2}$	$\frac{57}{120}$
30	S	$\frac{1}{5}$, $\frac{1}{5}$	3	3	$\frac{2}{5}$
34	S	$\frac{1}{5}$, $\frac{1}{5}$, $\frac{1}{5}$	2–3	$4\frac{1}{2}$	$\frac{3}{5}$
31	S	$\frac{1}{6}$, $\frac{1}{3}$	7	5	$\frac{1}{2}$
35	S	$\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{3}$	8	$4\frac{1}{2}$	$\frac{19}{30}$
37	S	$\frac{1}{20}$, $\frac{1}{10}$, $\frac{3}{20}$, $\frac{1}{5}$, $\frac{1}{4}$	3–4	$3\frac{1}{2}$	$\frac{3}{4}$
36	S	40 injections of $\frac{1}{100}$ each	1	Survived	$\frac{2}{5}$
42	P	$\frac{1}{100}$, $\frac{1}{50}$, $\frac{1}{30}$, $\frac{1}{25}$, $\frac{1}{12}$, $\frac{1}{6}$, $\frac{1}{3}$, $\frac{2}{3}$, 1	1–3	$1\frac{1}{2}$	$2\frac{1}{3}$
41	P	$\frac{1}{10}$, $\frac{1}{8}$, $\frac{1}{6}$	7	$8\frac{1}{2}$	$\frac{57}{120}$
62	I	$\frac{1}{10}$, $\frac{1}{8}$	8	$2\frac{1}{2}$	$\frac{1}{5}$

S=subcutaneous. P=intraperitoneal. I=intravenous.

Preliminary treatment with large quantities of antitoxin has apparently but little power to confer a lasting passive immunity. This is shown by an experiment in which two rabbits were each given 3,000 units of diphtheria antitoxin in three equal doses on alternate days. After one week the animals were bled and the serum tested for antitoxin. None could be demonstrated. Each rabbit was then given ten rabbit M. L. D.s. of toxin. One died the following day, the other survived, and was given twenty times the M. L. D. a week later. It was found dead the next morning. The experiment shows, however, that the administration of antitoxin, notwithstanding its disappearance from the blood, confers a little immunity.* This method of immunization is of course quite different physiologically from that in which repeated doses of the toxin itself are employed.

Not merely has it been found impossible, as shown above, to produce any artificial immunity by repeated injection of toxin, but the rabbits were found to develop, in some cases, a supersensitiveness or hypersusceptibility to the toxin which caused their death upon administration of sublethal doses (cf. Table 8). This occurred with intraperitoneal and intravenous as well as subcutaneous injections.

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* Experiments are planned to determine accurately how high this degree of immunity is, what ratio it bears to the amount of antitoxin administered, and how long antitoxin can be detected in the serum.

That this phenomenon is a true hypersusceptibility and not a mere cumulative action, such as discussed in connection with the white rat, is shown by the fact that a number of these rabbits died before the sum of all the doses administered had reached the lethal quantity. Inasmuch, therefore, as no cumulative action was concerned in these cases, there is justification for the assumption that it was absent in the other cases as well, even where the sum of all the doses administered exceeded the lethal quantity, and that the rabbit was killed by the last dose given rather than by a summation of all the quantities injected.

Hypersusceptibility to toxin injections has been observed in other animals to a much more marked degree than occurs in the case of the rabbit to diphtheria toxin, as shown by experiments described above. Behring and Kitashima[20] report that guinea-pigs, started with the injection of 1/400,000 M. L. D. of diphtheria toxin, die upon receiving from 1/800 to 1/700 M. L. D., although the sum of all the injections amounted to but 1/400 M. L. D. This result is of course far more pronounced than that of the above experiment on rabbits. The authors show further that evidences of hypersusceptibility to this toxin can be obtained in horses and apes, and also cite the case of a horse that died of diphtheria intoxication during the process of immunization, although the blood possessed antitoxin. Rist[21] injected small quantities of dried diphtheria bacilli into guinea-pigs and found that the first dose produced but a transient illness, the second a more protracted one, and the third caused death. Brieger[22] reports the case of a goat that died of tetanus toxin although immunized to such an extent that considerable quantities of antitoxin were found in the blood and milk. Knorr[23] was able to produce so great a hypersusceptibility in guinea-pigs toward tetanus toxin that even 1/50 M. L. D. might cause death. It is interesting to note that his experiments on rabbits not merely produced no hypersusceptibility but immunized them to the extent of withstanding five times the M. L. D. of tetanus toxin. Finally there is the so-called "paradoxical phenomenon of Kretz," quoted by Wassermann,[24] that while normal animals do not react to a mixture of toxin and antitoxin in certain proportions, animals previously immunized to that toxin will react to the mixture, due, no doubt, to incomplete neutralization of the toxin by the antitoxin, leaving but a sublethal quantity of toxin free which is too small to affect normal animals but will injure those made hypersusceptible.

In regard to the results shown in Table 8, the first thing to be done is to determine which factor in the procedure causes the phenomenon. To see if the effect of the mere number of injections could be responsible, a rabbit was given, for over a month, daily injections of 1.5 c.c. of sterile physiological salt solution, as it was to this volume that all toxin injections in the hypersusceptibility series were diluted with salt solution. The animal not merely showed no inconvenience from the procedure, but gained in weight quite noticeably, so it is evident that the mere effect of the repeated injections cannot account for any part of the findings. The next element to be considered was the broth conveying the toxin. As this may have caused an anaphylaxis, similar to that exhibited toward sera, corpuscle extractives, etc., a rabbit was given twelve injections of 0.5 c.c. of Theobald Smith's[25] diphtheria infusion broth (which was used for the production of the toxin) plus 1 c.c. of salt solution. The animal thrived and gained weight during the experiment. As the hypersusceptibility to foreign albumins is often most marked following the repeated injection of very small quantities, another animal was submitted to the same procedure, except that only 0.1 c.c. of the infusion broth and 1.4 c.c. of salt solution were given. This animal also gained weight. Since, then, neither the broth nor the effect of the repeated injections is able to lower the vitality of the animal, the only alternative is that the hypersusceptibility is to the toxin alone.

Several theories have been advanced to explain the phenomenon of hypersusceptibility, when it occurs following injections of foreign sera (anaphylaxis, serum disease). Thus Bail[26] holds that repeated injections of a serum causes the production of a substance which prevents the leucocytes from fulfilling their normal rôle of protection. Richet[27] believes that serum contains two bodies, one of which tends toward the production of immunity, and the other of the phenomenon of hypersusceptibility in the animal injected. Courmont[28] advances the theory that the first injection causes some protective substance to be absorbed, so that when the second injection is made there is nothing to neutralize the toxic principle in the serum. This theory is elaborated by Currie,[29] who holds that the active principle in the injected serum gives rise to an antibody, or rather causes one to be produced by the animal, union with which forms the true toxic principle. When but one injection of serum is made, the antibody is produced so gradually that the body is either able to eliminate the combined form or else to produce another antibody to neutralize the toxic compound resulting from the serum substance and the first antibody. If a second injection of serum is made, however, enough of the first antibody is left over from the effects of the first injection to produce intoxication, either because there is too much to be eliminated quickly or because the second antibody has disappeared from the body during the interval. Wright,[30] observing a fall and then a rise in the bactericidal power of the blood after inoculation with vaccine, holds that hypersusceptibility may be explained by the injection of a sufficient quantity of the immunizing agent during the negative phase to produce a cumulation.

As shown above, however, cumulation cannot be the explanation of hypersusceptibility to toxin, and the experiments of Behring and Kitashima[20] on guinea-pigs corroborate this. That it does not apply even to serum hypersusceptibility is seen from the experiments of Otto[31] and of Rosenau and Anderson,[32] so that Wright's hypothesis cannot be accepted. Since Otto and Dungern[33] have shown that precipitins do not appear in the blood at the time of hypersusceptibility, this explanation also must be discarded, and, as pointed out by Currie,[29] the theories of Courmont, Richet, and Bail fail to take account of the time interval between injections, a factor apparently quite significant, as Table 8 indicates that little or no hypersusceptibility was shown by the animals injected at short intervals. While data upon this point are as yet incomplete, a comparison of Rabbits 29 and 41 in Table 8 shows that an interval of three or four days is just as effective in producing a hypersusceptibility as a longer period, while the records of Rabbits 30 and 34 show that two days is not as effective as three. These assumptions appear to be borne out by the other animals, though not in a manner so easy to interpret.

Although Currie's theory may account for the phenomenon when it is occasioned by repeated injections of serum, it is apparent that it cannot hold for the phenomenon elicited by diphtheria toxin. The mere fact that a single dose of toxin, if sufficiently large, is capable of killing a rabbit in a few hours shows that it is not dependent for its toxicity upon union with a slow-forming antibody, which is the basis of Currie's hypothesis.

The explanation of the phenomenon, as regards supersensitization to toxin, appears to me to be much simpler. If we look upon the body cells as belonging to three types, nervous tissue cells, essential to life, which are able to bind the toxin, cells not essential to life which are also able to bind the toxin but have a higher affinity for the toxin than the nervous tissue, and finally neutral cells which are inert in respect to the toxin, the question will be easier of solution.

Because of greater affinity the vitally-unessential cells of the second class will take up all the toxin introduced into the animal as far as their saturation limit, and of a toxin dose within this limit none is able to reach the vital nervous tissue. A lethal dose of toxin is one which so exceeds this limit that enough toxin is left over to unite with enough nervous tissue to cause death. If we let 1 equal the quantity of toxin sufficing to destroy enough of the nervous tissue, when allowed to reach it, to kill the animal, and let 9 equal the amount of toxin which the less essential, but chemically more active, cells can take up, then the injection of 9 units of toxin will injure the latter cells, but since they are not necessary to life the animal will not suffer seriously. A lethal dose would be $9 + 1 = 10$ units, as this would leave 1 unit able to reach the central nervous system. If a 9-units dose be given, the protective cells are destroyed, or at least so injured (perhaps by loss of the proper receptors) that the nervous tissue is left without any safeguard and the administration of a single unit would kill the animal. This condition lasts until the protective cells or their damaged receptors are regenerated, a process which, as the foregoing data indicates, takes place very slowly. Hypersusceptibility, or death from doses amounting to less than ten units, can be explained by analogy with well-known pathological processes. Suppose an initial dose of one unit be given a normal animal, one-ninth of the protective tissue receptors are immediately bound and destroyed, and their degeneration may cause the degeneration of another two-ninths of these receptors in the same manner as necrosis of a certain number of cells in an organ is often followed by spreading necrosis of a large additional area, or else it may be caused by interference with nutrition, etc., after the manner in which occlusion of a blood vessel supplying a region produces an anemic infarct. A period sufficient for the development of this degeneration but insufficient for regeneration being allowed to intervene, a second dose of toxin amounting to two of the hypothetical units can, by uniting with two-ninths of the remaining protective tissue receptors, cause the degeneration and sloughing-off of, say, another three-ninths of these receptors, leaving but one-ninth of the protective receptors left to guard the vital central nervous system. After a like interval a third dose of two units is given, one-half of which is bound by the remaining one-ninth of the protective tissue receptors, while the other half is free to unite with the receptors of the nervous system and thus kill the animal, although only one-half as much toxin has been given in all the doses as is required to kill the animal at a single administration. If objection be offered to the "receptor" terminology employed above, the same hypothesis will hold by assuming an excretory function for the protective cells. It is known that small quantities of certain drugs are able to produce a slow-forming inflammation and degeneration of the organ which excretes them, as the kidney. Thus a large dose is eliminated after injection, but after a while so much harm has been done by the resulting inflammation that a second small dose cannot be eliminated and produces toxic effects. Inasmuch as a smaller dose can produce the same amount of inflammation as the larger, the same amount of poisoning would be produced by a smaller original dose. Applying this to the effect of toxin on rabbits, we might assume that the protective cells are normally able to eliminate in some way, perhaps after a change in chemical character, certain amounts of toxin. The process, however, is so injurious to them that a degenerative change soon develops in the part of the cell carrying out this function which renders it incapable of repeating the process for a considerable time. Then when a fraction of the lethal dose of toxin is given it is eliminated or changed by these cells so that it cannot reach the central nervous system, but in the course of three or four days there appears a degeneration of the eliminating mechanism in these cells lasting some weeks at least, so that when another small fraction is given it cannot be eliminated and thus gets to the nervous tissue, which we know to be susceptible to very small amounts of toxin, and kills the animal. When a single large dose of toxin, sufficient to kill the animal, is given, the mechanism of action is different because the eliminating apparatus is in working order. Death results, however, because the amount of toxin administered is too large to be gotten rid of with sufficient rapidity to prevent the small moiety sufficient for that purpose from uniting with the nervous system.

It seems to me that this theory avoids the objections raised to the other hypotheses cited, in that it explains both the significance of the time interval necessary between injections, and the mechanism of death from the administration of a single large dose.

SUMMARY.

The foregoing is a study of the reactions of a naturally immune mammal, the white rat, and one naturally susceptible, the rabbit, toward diphtheria toxin.

Experiments have shown that the rat is not absolutely immune but that it is killed in four days by 3,500 times the guinea-pig lethal dose, corrected to weight, when injected subcutaneously.

The resistance of half-grown rats is half the above figure, even when corrected for weight.

The intraperitoneal M. L. D. for the rat is 1,200 times the guinea-pig subcutaneous M. L. D.

A local semi-specific alopecia occurs at the site of subcutaneous injection.

I have been able to exalt the natural resistance of the rat by repeated injections of increasing doses of toxin.

No cumulative action or hypersusceptibility is exhibited.

The immunity of the rat is not due to free antitoxins in the blood.

The fate of toxin injected into the rat has been traced in detail, and a theory advanced to explain the mechanism of the rat's immunity.

Subcutaneous, intraperitoneal, and intravenous minimum lethal doses for the rabbit have been ascertained, using the guinea-pig as a standard, and an hypothesis formulated to explain the resistance mechanism.

It is found that rabbits exhibit hypersusceptibility to injections of toxin, whether subcutaneous, intraperitoneal, or intravenous. A theory is advanced to explain this phenomenon.

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REFERENCES.

1. ROUX AND YERSIN. *Ann. de l'Inst. Past.*, 1888, 2, p. 661.
2. COBBETT. *Brit. Med. Jour.*, 1899, 1, p. 902.
3. HITCHENS. *Jour. Exp. Med.*, 1906, 8, p. 647.
4. KUPRIANOW. *Centralbl. f. Bakt.*, 1894, 16, p. 415.
5. BEHRING. *Deutsche med. Wchnschr.*, 1890, 16, p. 1145.
6. ROUX AND BORREL. *Ann. de l'Inst. Past.*, 1898, 12, p. 225.
7. COBBETT. *Brit. Med. Jour.*, 1900, 2, p. 21.
8. BRUNNER. *Arch. des sci. biol. de St. Petersb.*, 1898, 6, p. 202.
9. ASAKAWA. *Centralbl. f. Bakt.*, 1898, 24, pp. 168,234.

10. VAILLARD. *Ann. de l'Inst. Past.*, 1892, 6, 229.
11. METCHNIKOFF. *Ann. de l'Inst. Past.*, 1897, 11, p. 808.
12. ——. *L'Immunité*, Paris, 1901, pp. 343 ff.
13. ROSENAU AND ANDERSON. *Jour. Infect. Dis.*, 1907, 4, p. 1.
14. WASSERMANN AND TAKAKI. *Berl. klin. Wchnschr.*, 1898, 35, p. 5.
15. SALTER. *Lancet*, 1898, 1, p. 152.
16. ROUX AND YERSIN. *Ann. de l'Inst. Past.*, 1897, 11, p. 808.
17. KNORR, cited by Dieudonné. *Immunität, Schutzimpfung u. Serumtherapie*, Leipzig, 1905.
18. DE CROLY. *Arch. de pharmacodynamie*, 1897, 3, p. 61.
19. BOMSTEIN. *Centralbl. f. Bakt.*, 1898, 23, p. 785.
20. BEHRING AND KITASHIMA. *Berl. klin. Wchnschr.*, 1901, 38, p. 157.
21. RIST. *Comptes rend. de la Soc. biol.*, 1903, 55, p. 978.
22. BRIEGER. *Ztschr. f. Hyg.*, 1895, 19, p. 101.
23. KNORR, cited by Metchnikoff. *L'Immunité*, p. 394.
24. WASSERMANN, in Kolle u. Wassermann. *Hdbch. d. path. Mikroörg.*, Jena, 1903.
25. THEOBALD SMITH. *Jour. Exp. Med.*, 1899, 4, p. 373.
26. BAIL, cited by Pirquet and Schick. *Münch. med. Wchnschr.*, 1906, 53, p. 67.
27. RICHET. *Ibid.*
28. COURMONT. *Ibid.*
29. CURRIE. *Jour. of Hyg.*, 1907, 7, p. 35.
30. WRIGHT. *Brit. Med. Jour.*, 1903, 1, p. 1069.
31. OTTO. *v. Leuthold-Gedenkschrift*, 1905, 1.
32. ROSENAU AND ANDERSON. *Jour. Med. Res.*, 1906. 15, p. 179.
33. DUNGERN. *Die Antikörper*, Jena, 1903.